



# Imatinib sensitizes endometrial cancer cells to cisplatin by targeting CD117-positive growth-competent cells



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## ABSTRACT

The use of molecular target therapy has not been established for endometrial cancer. The present study investigated the potential therapeutic strategy of targeting CD117-positive cancer cells as a novel molecular target therapy. FACS-sorted CD117<sup>+</sup> cells isolated from endometrial cancer cell lines (Ishikawa or MFE280 cells) exhibited higher proliferative capacity *in vitro* and colony forming activity on soft agar, and decreased sensitivity to cisplatin, compared to CD117<sup>−</sup> cells. Immunohistochemical analyses with surgical specimens of endometrial cancers showed that high CD117 expression was tightly linked to advanced FIGO stages, myometrial invasion and histological grade, and was significantly associated with poor overall survival and relapse-free survival (Kaplan–Meier analysis;  $p < 0.001$ , log-rank test). The Cox-regression hazard model identified high CD117 expression to be an independent prognostic factor for survival ( $p < 0.05$ ). *In vitro* assay confirmed that stem cell factor (SCF), a ligand of CD117, was produced specifically in CD117<sup>+</sup> cells of endometrial cancer, and the colony-forming activity were abrogated by adding anti-SCF antibody, indicating an SCF-dependent growth property. Imatinib was confirmed to selectively target CD117<sup>+</sup> cells *in vitro*, and synergistically enhanced the anti-tumor effect of low dose cisplatin *in vivo*, which showed only modest effects when used as a single use. These findings suggest that CD117 can be a marker of aggressive behavior of cells as well as an independent prognostic marker in endometrial cancer. Targeting of the SCF/CD117 axis by imatinib sensitized endometrial cancer cells to cisplatin, proposing a novel therapeutic strategy for this tumor type.

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## 1. Introduction

Endometrial cancer is the third most common gynecologic cancer in Japan, and its morbidity and mortality have dramatically increased in the past 30 years [Matsuda, 2012, Cancer Incidence and Incidence Rates in Japan in 2006: Based on Data from 15 Population-based Cancer Registries in the Monitoring of Cancer Incidence in Japan (MCIJ) Project]. Although the majority of patients with early stage endometrial cancer are cured through surgery, patients with advanced stage cancer or recurrent lesions are not well-controlled, even with radiation and chemotherapy treatment, for which new molecular target therapies are urgently needed.

The present study aimed to identify molecular targets for endometrial cancer. The emphasis of the study was on CD117 (c-kit), which is a cell-surface receptor tyrosine kinase type III that triggers cell survival, proliferation, and differentiation, or other cellular functions when stimulated by stem cell factor (SCF) [3,16,21,25].

The reason for focusing on this molecule was that the specific inhibitor has been established and available, and its effects have been analyzed in other tumor types. CD117 has been known as a proto-oncogene, and its gene mutation and overexpression have been observed in gastrointestinal stromal tumors (GIST) [12], seminoma [17] and leukemia [11]. Molecular target therapy using imatinib mesylate, which blocks the tyrosine kinase activity of CD117, is an effective treatment for GIST [6]. Recent studies have reported interesting findings that CD117 is a potential marker of cancer stem cells in leukemia [7], lung cancer [13] and ovarian cancer [27]. This background prompted us to investigate the significance of CD117 expression in endometrial cancer. We explored the potential utility of targeting CD117 by imatinib as a novel molecular therapy, especially when combined with cisplatin, a key drug for conventional chemotherapy for this tumor type.

Our data showed that CD117 can be a marker of high proliferative capacity and a prognostic factor of endometrial cancer. Furthermore, we report that the SCF/CD117 axis may be a potential therapeutic target for endometrial cancer through its action of sensitizing endometrial cancer cells to cisplatin.

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## 2. Materials and methods

### 2.1. Cell culture

The human endometrial cancer cell lines, Ishikawa and MFE280 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin, and 100 IU/mL penicillin in 5% CO<sub>2</sub> incubated at 37 °C.

### 2.2. Flow cytometry

Cells were incubated at 4 °C in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 2 mmol/L EDTA with phycoerythrin-conjugated anti-human CD117-PE antibody (BD Biosciences, San Diego, CA, USA), and analyzed by flow cytometry (JSAN desktop cell sorter and App San software; Bay Bioscience Co, Ltd., Kobe, Japan). CD117<sup>+</sup> and CD117<sup>-</sup> cells were sorted using a JSAN desktop cell sorter. Isotype-matched mouse immunoglobulin G (IgG) was used as a control.

### 2.3. Cell proliferation and chemosensitivity assay

The cells were seeded at a density of  $3 \times 10^4$  or  $1 \times 10^5$  per well in 24-well tissue culture plates in DMEM with 10% FBS in 5% CO<sub>2</sub> at 37 °C. The cell number was counted using a hemocytometer with trypan blue staining at 2, 4, 6, and 8 days after the seeding. For the chemosensitivity assay, cells were seeded in 96-well plates at  $2 \times 10^3$  cells/well, in DMEM medium with 10% FBS and incubated at 37 °C for 24 h before adding drug. Indicated concentrations of cisplatin (provided by Bristol Pharmaceuticals, YK, Tokyo, Japan) were added in 0.1 mL medium, and the cells were cultured at 37 °C for 72 h. Then, the WST-1 assay (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used as described by the manufacturer.

### 2.4. In vitro colony formation assay

In vitro colony formation capacity was evaluated by a clonogenic assay. Diluted single cells were plated onto 60-mm tissue culture dishes and incubated in 5% CO<sub>2</sub> at 37 °C for 8 days. Colonies larger than 250 µm in diameter were counted after Giemsa staining.

### 2.5. Soft agar colony formation assay

A total of  $2 \times 10^4$  single cells were plated in 60-mm dishes containing 0.33% soft agar in DMEM supplemented with 10% FBS on top of 0.5% base agar in DMEM supplemented with 10% FBS. Cultures were maintained in a humidified 37 °C incubator. Colonies larger than 0.25 mm were counted after incubation for 2 weeks.

### 2.6. Small interfering RNA (siRNA) treatment

siRNA against CD117 (sc-29225) or the negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Cells were transfected with siRNA by the Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction.

### 2.7. Cell cycle analysis

Cells were fixed with 70% ethanol overnight at –20 °C, suspended in 0.1% Triton X-100 (in PBS) containing 25 µg/mL RNase (Sigma) for 30 min at 37 °C, and then incubated with 50 µg/mL propidium iodide (Invitrogen) to stain nuclear DNA for 30 min on ice. DNA content was analyzed using a Beckman Coulter Gallios (Beckman Coulter, Brea, CA, USA). Data collected from the experiments were analyzed using Kaluza analysis software (Beckman Coulter).

### 2.8. Patients and clinical samples

A total of 94 patients with endometrioid-type endometrial cancer (aged 32–81 years; mean age, 58.8 years) treated at the Department of Obstetrics and Gynecology, Kanazawa University Hospital between 1999 and 2005, were included in this study. All patients basically underwent simple abdominal hysterectomy (or radical abdominal hysterectomy), bilateral salpingo-oophorectomy and pelvic lymphadenectomy. Staging of all tumors was conducted according to the International Federation of Gynecology and Obstetrics (FIGO 1998) criteria. Among the 94 study patients, 63.8% of patients were diagnosed at FIGO stage I and 14.9% of patients were diagnosed at FIGO stage II, whereas 13.8% of patients were diagnosed at FIGO stage III and 2.1% of patients were diagnosed at FIGO stage IV. Based on histological examination, grade G1 was observed in 61.7%, G2 in 16.0%, and G3 in 22.3% of patients. Adjuvant radiation therapy of the whole pelvis with an external beam was administered at a total dose of 50.4 Gy in 31 patients with risk factors for recurrence; i.e., deep myometrial invasion (>1/2), grade 3 tumor, cervical extension, or extrauterine disease. Patients with positive peritoneal cytology, with lesions that extended outside the pelvis or with lymphnode metastasis were administered

adjuvant chemotherapy in combination with Cisplatin (75 mg/m<sup>2</sup>), Doxorubicin (50 mg/m<sup>2</sup>) and Cyclophosphamide (500 mg/m<sup>2</sup>) in 4 cycles from 1995 to 2000, and in combination with paclitaxel (180 mg/m<sup>2</sup>) and carboplatin (AUC = 5) in 4–6 cycles since 2001. Since 2004, patients in FIGO stage III have been routinely administered adjuvant chemotherapy in combination with paclitaxel (180 mg/m<sup>2</sup>) and carboplatin (AUC = 5) in 4–6 cycles. A total of 20 patients received platinum-based chemotherapy.

The patients were informed and gave their consent to using tumor samples of their hysterectomy specimens for the study. The protocol was previously approved by the Ethics Committee of Kanazawa University.

### 2.9. Immunohistochemistry

Immunohistochemical analysis was performed on 4-µm-thick, formalin-fixed, paraffin-embedded tissue sections. After the specimens were deparaffinized in xylene and graded alcohols, tissue sections prepared for antibody application were pretreated with heat-induced epitope retrieval performed in a water bath with buffer (pH 6.0, Target Retrieval, Dako, Carpinteria, CA, USA) for 16 h at 60 °C. Afterward, endogenous peroxidase was blocked by immersing the sections in 3% H<sub>2</sub>O<sub>2</sub>-methanol for 10 min. After blocking with goat serum, the rabbit polyclonal anti-human antibody c-kit (CD117, Dako) at 1:100 dilution was applied and incubated for 30 min at room temperature. The sections were stained for 30 min at room temperature with the EnVision + HRP Kit (Dako). The reaction products were developed by immersing the sections in a 3,3'-diaminobenzidine tetrahydrochloride solution. Nuclei were lightly counterstained with hematoxylin. CD117 expression was assessed semi-quantitatively by two independent investigators as follows: negative, weak (up to 1% of cells stained), moderate (1–10%) or strong (>10%). Immunohistochemically CD117-positive GIST tissue was used as positive control.

### 2.10. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (from approximately 10<sup>6</sup> cells) was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Isolated RNA (2 µg) was reverse transcribed with the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. Complementary DNA was amplified using Taq polymerase (Nippon Gene, Tokyo, Japan). Primers and PCR conditions are listed in Table 1. PCR products were separated and visualized on 2% agarose gel containing ethidium bromide. The following primers were used: 5'-GGTGGCAAATCTTC-CAAAG-3' and 5'-TCITTCACGCACTCCACAAG-3' for SCF; 5'-GGCATCACGGTGACTT-CAAT-3' and 5'-GGTTTGGGGAATGCTTCATA-3' for CD117; 5'-CTCAGACACCATGGGG-AAGGTGA-3' and 5'-ATGATCTTGAGGCTGTGTTCATA-3' for GAPDH.

### 2.11. Enzyme-linked immunosorbent assay (ELISA)

The cells ( $2 \times 10^5$ ) were plated onto 60-mm tissue culture dishes, and cultured in DMEM serum-free medium with PBS control, IgG control (1.0 µg/mL) (R&D Systems, Minneapolis, MN, USA) or anti-SCF (1.0 µg/mL) (R&D Systems) for 1–4 days.

**Table 1**

Correlation between CD117 expression and clinicopathological characteristics of endometrial cancer.

Variable	CD117 expression		P value (Fisher's test)
	Low (n = 85)	High (n = 9)	
Age (y)			
<60 (n = 54)	50	4	0.3144
≥60 (n = 40)	35	5	
FIGO stage			
I, II (n = 78)	74	5	0.0336
III, IV (n = 16)	11	4	
Lymph node metastasis			
Negative (n = 87)	78	9	0.4822
Positive (n = 7)	7	0	
Depth (myometrial invasion)			
<1/2 (n = 66)	63	3	0.0188
≥1/2 (n = 28)	22	6	
Histopathologic degree of differentiation			
Grade 1, 2 (n = 73)	69	4	0.0243
Grade 3 (n = 21)	16	5	
Menopause			
Peri-/pre- (n = 68)	60	8	0.227
Post- (n = 26)	25	1	
Body mass index (kg/m <sup>2</sup> )			
<25 (n = 58)	53	5	0.4753
≥25 (n = 36)	32	4	

Conditioned medium was respectively collected and stored at  $-20^{\circ}\text{C}$  until tested. The amounts of SCF in cell culture supernatant samples were quantified by ELISA kits (R&D Systems) according to the manufacturer's protocol. Standard curves were obtained by plotting known concentrations of SCF versus absorbance. Results are presented as concentrations in pg/mL.

### 2.12. Xenograft models

Female 6–8-week-old Balb/c nude mouse nu/nu (Charles River, Yokohama, Japan) were housed under specific pathogen-free conditions. Animal care was conducted according to institutional guidelines. Mice were inoculated subcutaneously into the right flanks with  $1 \times 10^7$  Ishikawa cells or  $1.5 \times 10^7$  MFE280 cells. After tumor cell inoculation, mice were randomly treated with PBS, cisplatin, imatinib, or the combination of cisplatin with imatinib. Each group consisted of 4 mice (for Ishikawa cells) or 6 mice (for MFE280 cells). Cisplatin (2 mg/kg for Ishikawa cells or 0.5 mg/kg for MFE 280 cells), imatinib (30 mg/kg) and PBS were administered intraperitoneally on Days 1, 3, and 5, Days 1, 2, 3, 4, and 5 or Days 2 and 4, respectively. Tumor diameters were measured every week with calipers; tumor volume in cubic millimeters was calculated as  $\text{volume} = (\text{short diameter})^2 \times \text{long diameter}/2$ .

### 2.13. Statistical analysis

Statistical analyses were performed using the statistical software package Stat-View version 5.0 (Abacus Concepts, Berkeley, CA, USA). We used Student's *t*-test for 2 groups or Tukey's test for more than 2 groups for *in vitro* experiments. The  $\chi^2$  test was used to evaluate the relationship between categorical variables in immunohistochemistry assays. Survival analysis was performed using the Kaplan–Meier method, and the significance between survival curves was determined using the log-rank test. Multivariate survival analysis was calculated by the Cox proportional hazards regression model in a stepwise manner for analyzing the independent prognostic factors. Probability values of  $<0.05$  were considered statistically significant.

## 3. Results

### 3.1. Growth behavior and clinicopathological characteristics of CD117<sup>+</sup> cells in endometrial cancer

We first examined the frequency of the CD117<sup>+</sup> subpopulation in 2 representative endometrial cancer cell lines, Ishikawa and MFE280 cells, by flow cytometric analysis using anti-human CD117 antibody (Supplementary Fig. 1). The CD117<sup>+</sup> subpopulation in the Ishikawa and MFE280 cell lines was approximately 2.3% and 78.7%, respectively. Since the percentage of CD117<sup>+</sup> cells was extremely high in MFE280 cells, we sought to confirm the expression profile of CD117 during the culture. Flow-cytometric analysis of FACS-sorted CD117<sup>+</sup> cells revealed that the ratio of CD117<sup>+</sup> cells became approximately 80% on day 6 after the sorting, returning to the initial population ratio. These findings show that 10–20% of CD117<sup>+</sup> cells change to CD117<sup>−</sup> cells in this cell type, maintaining high frequency of CD117<sup>+</sup> cells (Supplementary Fig. 2). The proliferative capacity and chemosensitivity of CD117<sup>+</sup> cells were evaluated *in vitro* and *in vivo* using the Ishikawa and MFE280 cell lines. Fluorescence-activated cell sorting (FACS)-sorted CD117<sup>+</sup> and CD117<sup>−</sup> cell populations were subjected to the growth assay and soft agar colony formation assay. The CD117<sup>+</sup> populations demonstrated increased proliferative and colony-forming activity compared with the CD117<sup>−</sup> populations (Fig. 1A and B). The siRNA-based knockdown of CD117 in FACS-sorted CD117<sup>+</sup> cells led to cell growth retardation and inhibition of colony-forming activity (Supplementary Fig. 3), indicating that CD117 expression at least partially contributes to growth-competent phenotype of endometrial cancer cells. Next, sorted CD117<sup>+</sup> and CD117<sup>−</sup> cells were treated with various concentrations of cisplatin, and cell viability was assessed using the WST-1 assay. CD117<sup>+</sup> cells isolated from the two cell lines showed significantly reduced sensitivity to cisplatin compared with CD117<sup>−</sup> cells (Fig. 1C), especially at lower doses. Higher doses conferred no significant difference in sensitivity, probably due to the toxic effects.

We next examined the clinicopathological significance of CD117 expression by immunohistochemistry using the 94 surgically removed tumors from the study patients. A total of 9 tumors

(9.5%) expressed high levels of CD117, while 85 tumors (90.5%) exhibited weak or no expression of CD117 (Fig. 2A). The correlation between CD117 expression and clinicopathologic characteristics of endometrial cancer was examined. CD117 expression was statistically correlated with myometrial invasion, FIGO stage and histologic grade, but not with patient age, body mass index (BMI), menopause or lymph node metastasis (Table 1). The 5-year prognosis for patients with high or low/no CD117 expression was compared; high CD117 expression was associated with poor clinical outcome in both overall survival (OS) (44% versus 86%,  $P < 0.001$ ) and relapse-free survival (RFS) (44% versus 89%,  $P < 0.0001$ ; Fig. 2B). A Cox proportional hazards analysis of OS and RFS was additionally performed, in which the values for CD117 expression, myometrial invasion and FIGO stage were considered as categorical variables; high CD117 expression was found to be an independent prognostic factor of RFS (Table 2). We further investigated the 5-year prognosis for 20 patients who received platinum-based adjuvant chemotherapy. As shown in Fig. 2C, patients with high CD117 expression ( $n = 5$ ) had significantly worse OS (20% versus 73%,  $P < 0.05$ ) and RFS (20% versus 73%,  $P < 0.05$ ), compared with those with low CD117 expression ( $n = 15$ ).

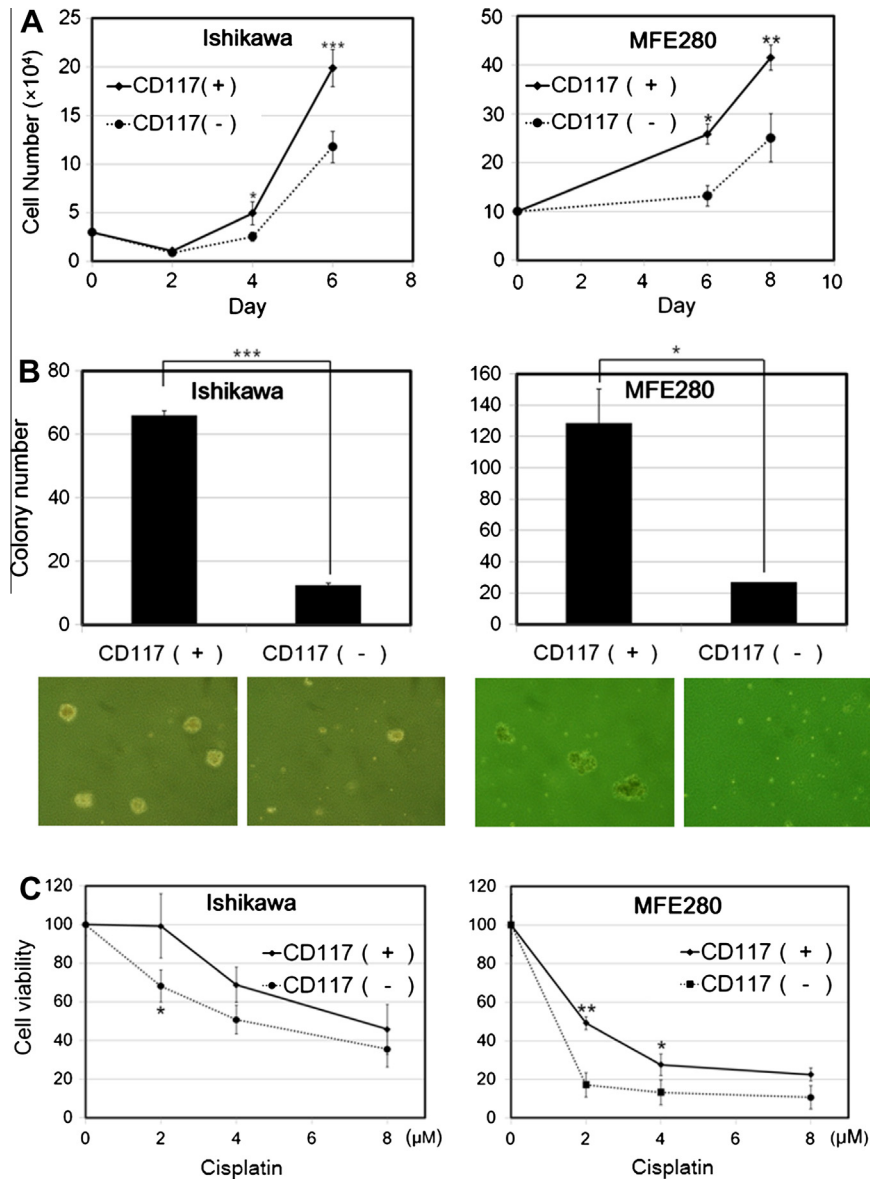
### 3.2. Expression and the role of SCF in endometrial cancer cells

We next examined the expression of stem cell factor (SCF), a CD117 ligand in CD117<sup>+</sup> or CD117<sup>−</sup>. The RT-PCR assay revealed markedly increased expression of SCF in CD117<sup>+</sup> Ishikawa cells compared with CD117<sup>−</sup> cells (Fig. 3A). Secretion of SCF in the Ishikawa cells was also confirmed by ELISA in growth media (Fig. 3B). The addition of anti-SCF antibody into the media significantly reduced the detectable levels of SCF, showing the assay specificity. We further tested the role of SCF in stem cell activity in Ishikawa cells. Clonogenic assay was performed with Ishikawa cells, in which anti-SCF or control IgG were added into the media. The addition of anti-SCF significantly inhibited the colony-forming activity of Ishikawa cells (Fig. 3C).

### 3.3. Imatinib suppresses the growth of CD117<sup>+</sup> cells in an SCF-dependent manner and sensitizes endometrial cancer cells to cisplatin

Because the CD117<sup>+</sup> cells had aggressive behavior, leading to poor prognosis, we sought to target the CD117<sup>+</sup> cells by using imatinib, a specific inhibitor for CD117. We first compared the sensitivity of imatinib to CD117<sup>+</sup> and CD117<sup>−</sup> cells in Ishikawa and MFE280 cells. After treatment with various concentrations of imatinib, cell viability was assessed using the WST-1 assay. As shown in Fig. 4A, CD117<sup>+</sup> cells showed significantly greater sensitivity to imatinib than CD117<sup>−</sup> cells in both cell types.

We next investigated whether the effect of imatinib is dependent on SCF. Cell growth assays were performed with CD117<sup>+</sup> and CD117<sup>−</sup> cells, in which they were treated with imatinib at 1  $\mu\text{M}$  in the presence or absence of anti-SCF antibody. As shown in Fig. 4B, imatinib inhibited the growth of CD117<sup>+</sup> cells in the absence of anti-SCF, and the addition of anti-SCF antibody significantly abrogated this effect in CD117<sup>+</sup> cells. CD117<sup>−</sup> cells did not respond to imatinib, regardless of the presence of anti-SCF antibody. In PBS control samples, the addition of anti-SCF antibody markedly inhibited the cell growth compared to that of control IgG antibody (compare right and left panel in Fig. 4B), confirming the role of SCF in cell growth. These findings indicate that imatinib suppresses the growth of CD117<sup>+</sup> cells in an SCF-dependent manner. We further examined whether this growth suppression by targeting CD117 is due to cell cycle arrest or induction of apoptosis. FACS-sorted CD117-positive cells were treated with siRNA-against CD117 or with Imatinib and analyzed their cell cycle profile by flow-cytometry. Both knockdown of CD117 and imatinib



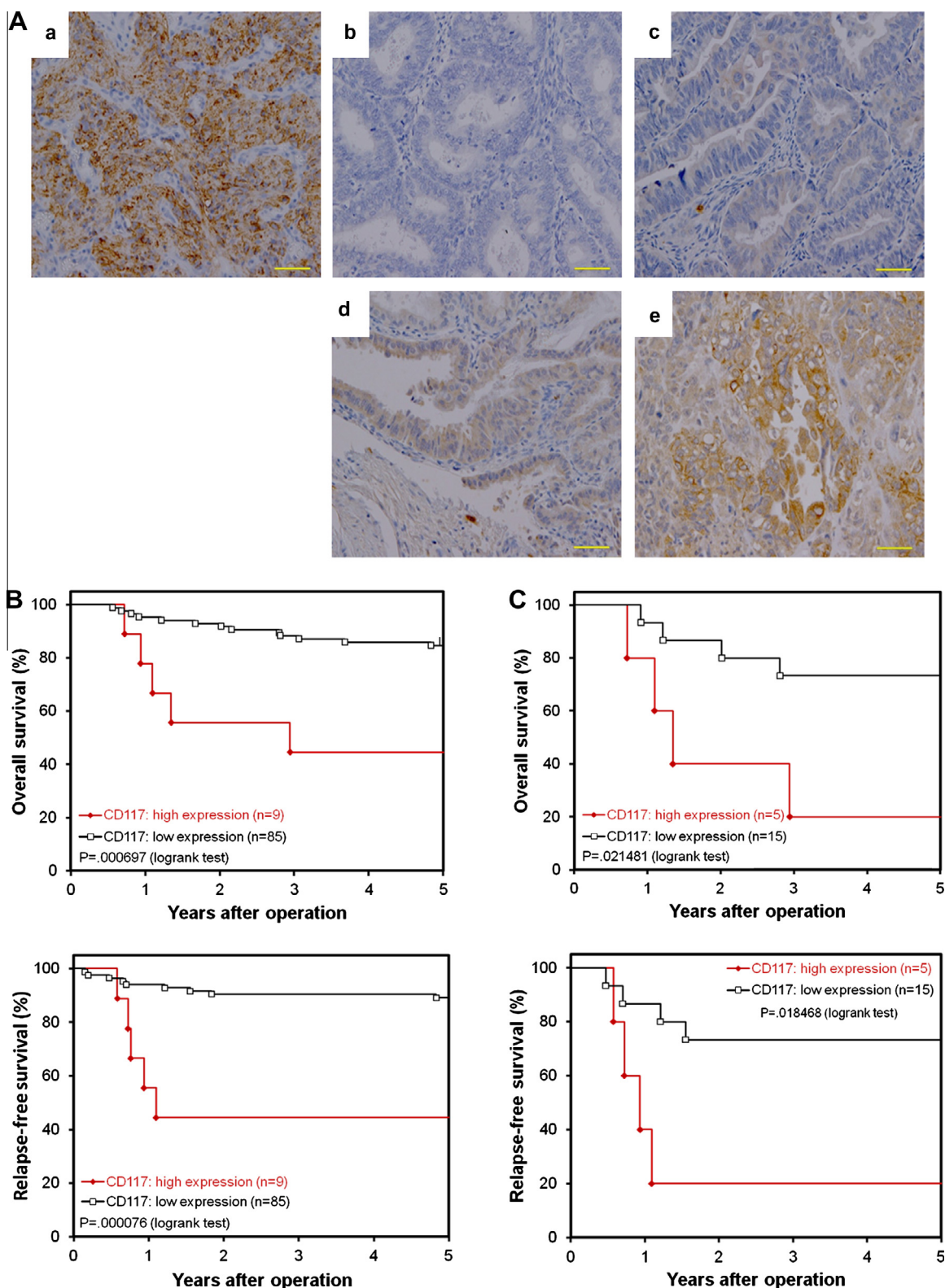
**Fig. 1.** *In vitro* proliferative and tumorigenic capacities of CD117<sup>+</sup> and CD117<sup>-</sup> endometrial cancer cells. (A) FACS-sorted CD117<sup>+</sup> or CD117<sup>-</sup> Ishikawa or MFE280 cells were grown in normal growth media for the indicated periods, and cell number was counted using a hemocytometer with trypan blue staining. (B) A total of  $2 \times 10^4$  FACS-sorted CD117<sup>+</sup> or CD117<sup>-</sup> Ishikawa or MFE280 cells were seeded on soft agar in 60-mm dishes, and colonies larger than 0.25 mm were counted after incubation for 2 weeks. Representative colonies are shown in the photographs. (C) A total of  $2 \times 10^3$  FACS-sorted CD117<sup>+</sup> or CD117<sup>-</sup> Ishikawa or MFE280 cells were seeded in a 96-well plate and exposed to various concentrations of cisplatin for 72 h in growth media. Cell viability was determined by WST-1 assay. Data are the mean  $\pm$  S.D. in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment led to the increase in G0/G1 fraction and decrease in S and G2/M fractions in Ishikawa cells (Supplementary Fig. 4A). However, there was a significant elevation of sub G0–G1 fraction in the treatment with imatinib but not with siRNA-against CD117. Therefore, the cell growth retardation by targeting CD117 appears to be due to both a decrease in cell proliferation and an induction of apoptosis. In MFE280 cells, the knockdown of CD117 or imatinib treatment led to the increase in G0/G1 fraction and decrease in S and G2/M fractions. There was no change in sub G0–G1 fraction (Supplementary Fig. 4B). These findings suggest that imatinib may target both CD117-related and -unrelated pathways in a cell-type dependent manner.

Our present findings suggested that CD117<sup>+</sup> cells were likely to be resistant to conventional chemotherapy, but relatively sensitive to imatinib. We therefore assumed that the combination of conventional chemotherapy with imatinib enhances antitumor

activity more than individual drug administration. To test this hypothesis, *in vitro* cell growth was monitored using Ishikawa or MFE280 cells in the absence or presence of imatinib and/or cisplatin for appropriate periods. As shown in Fig. 5A, only mild growth inhibition was observed with either imatinib or cisplatin alone, while the combination treatment exhibited an apparently potent cytotoxic effect. Soft agar colony formation assays were also performed to determine whether the combination of imatinib and cisplatin could affect tumorigenic growth of Ishikawa or MFE280 cells *in vitro*. Greater inhibition of colony formation was observed at 4 weeks in Ishikawa or MFE280 cells when the two drugs were combined, compared with either modality alone (Fig. 5B). We finally tested the *in vivo* effect of combination treatment using xenograft models with Ishikawa cells. The treatment groups consisted of the following: control (PBS intraperitoneally for 5 days), imatinib (30 mg/kg intraperitoneally for 5 days), cisplatin (2 mg/kg for





**Fig. 2.** Immunohistochemical analysis of CD117 expression in endometrial cancer. (A) Representative staining of CD117 in surgical specimens of endometrial cancer are shown. (a) A positive control for CD117 expression with a tumor specimen of a patient with GST. Cases with no expression (b), weak expression (<1.0% of immunostaining intensity of positive control) (c), moderate expression (1.0–10% of immunostaining intensity of positive control) (d), and strong expression (>10% of immunostaining intensity of positive control) (e). Bars indicate 100  $\mu$ m. (B) Kaplan–Meier analysis to evaluate the correlation of CD117 expression with overall and relapse-free survival of 94 patients with endometrial cancer. (C) Kaplan–Meier analysis to evaluate the correlation of CD117 expression with overall and relapse-free survival of 20 patients who received platinum-based adjuvant chemotherapy.

**Table 2**

Cox regression hazard model with prognostic factors.

Variables	Risk factor	Hazard ratio (95% confidence interval)	P
FIGO stage	>II	3.98 (1.22–13.01)	0.016
Depth	>1/2	4.37 (1.32–14.52)	0.01
CD117 expression	High	5.90 (1.54–22.58)	0.022

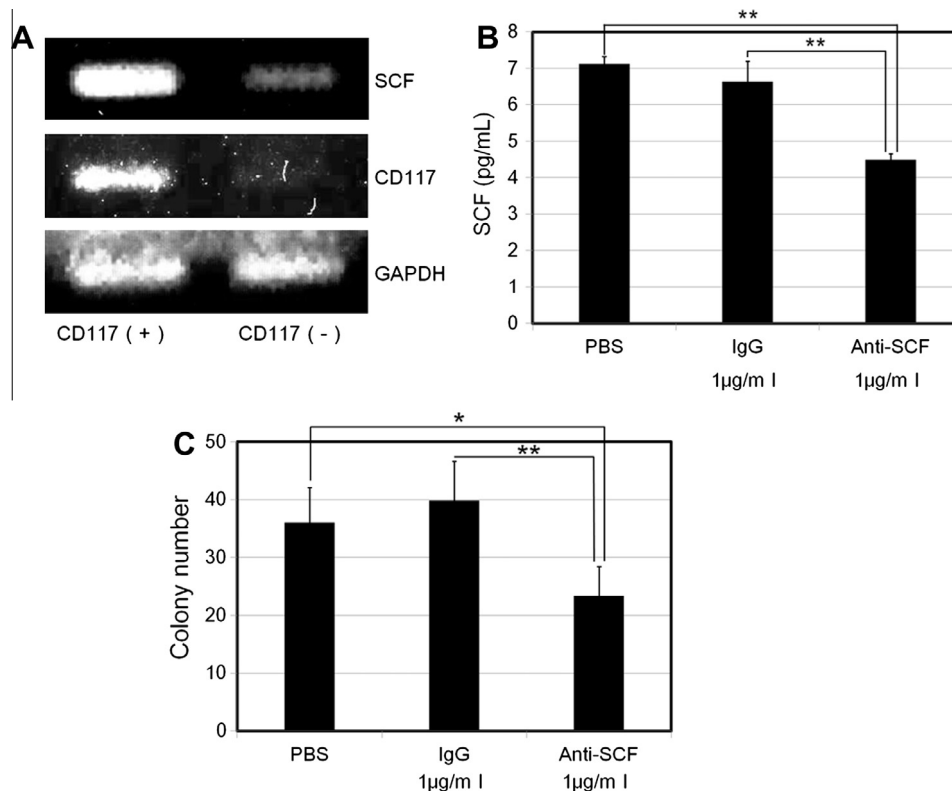
Ishikawa cells and 0.5 mg/kg for MFE280 cells intraperitoneally for 3 days), and combination of imatinib and cisplatin. Tumor volumes were monitored after inoculation of Ishikawa or MFE280 cells for appropriate periods. Under the concentrations of cisplatin or imatinib, at which only modest growth retardation was observed in a single use, the combination treatment showed significantly enhanced growth inhibition (Fig. 5C). Notably, we did not observe any clinical signs of toxicities in the mice treated with imatinib (30 mg/kg) and cisplatin (2 mg/kg for Ishikawa cells and 0.5 mg/kg for MFE280 cells). Taken together, these results suggest that imatinib at a relatively low concentration causes no significant adverse effects, and sensitizes endometrial cancer cells to cisplatin.

#### 4. Discussion

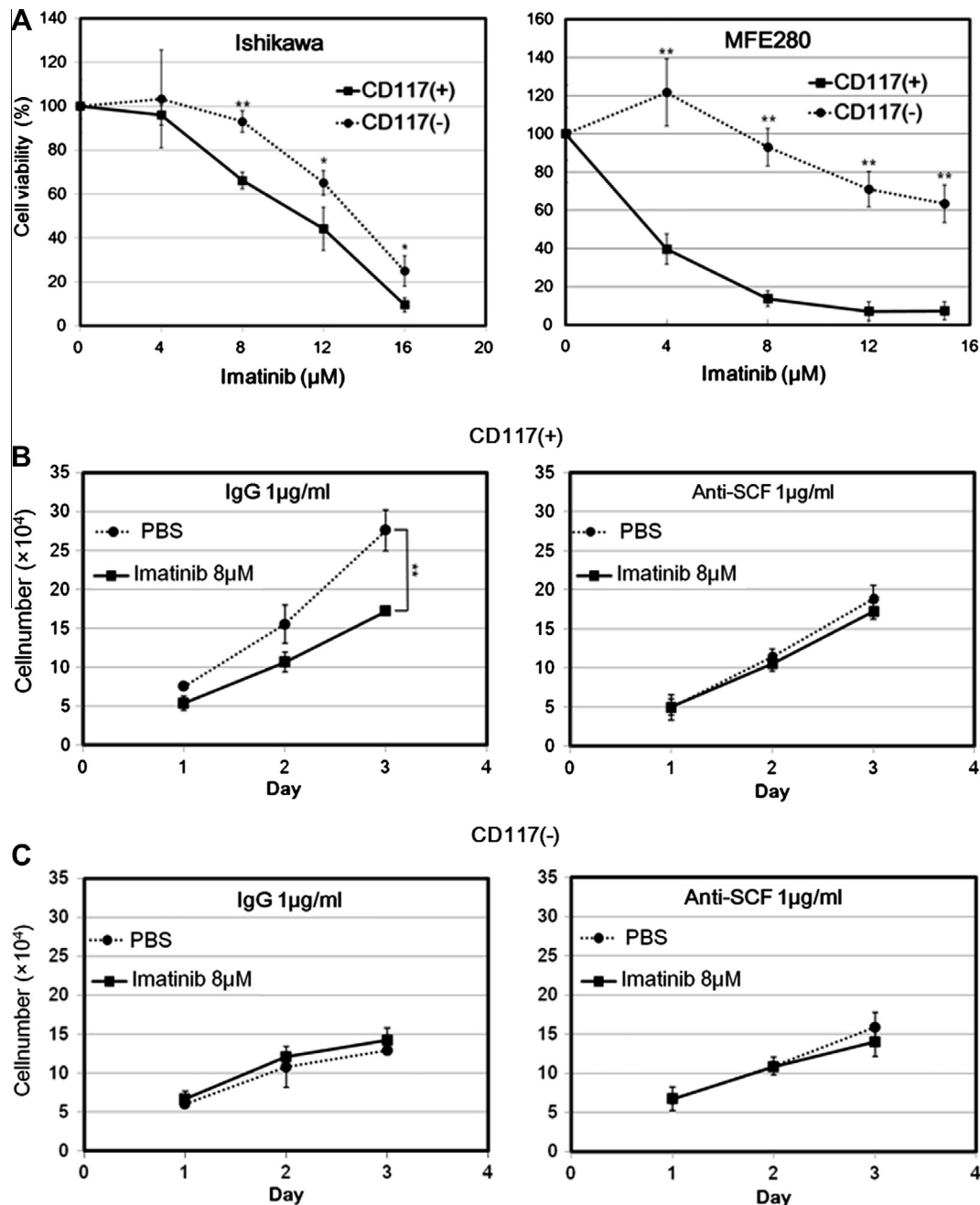
One interesting finding in the present study is that CD117 expression is an independent prognostic factor of this tumor type. Among the patients who received platinum-based adjuvant chemotherapy, those with high CD117 expression had significantly worse OS and RFS compared to those with low CD117 expression, supporting that CD117<sup>+</sup> cells have cancer stem cell-like features

with chemo-resistance. Consistent with our findings, cancer patients with CD117 overexpression and/or mutations are known to have poor prognosis in a variety of tumor types [9]. CD117 is a surface marker for embryonic stem cells, hematopoietic stem cells, and mesenchymal stem cells; it allows cells to remain in their undifferentiated state, and it confers the ability for self-renewal [1,2,15]. Accumulating evidence indicates that SCF is a mitogenic and angiogenic factor involved in carcinogenesis [16,24]. Binding SCF to CD117 results in the activation of its intrinsic tyrosine kinase activity and promotes tumor growth. This SCF-CD117 signaling loop may play a critical role in the aggressive behavior of tumors, affecting patient survival. The present study clearly demonstrated the superior efficacy of imatinib on CD117<sup>+</sup> cells. Since SCF is up-regulated in CD117<sup>+</sup> cells, and the blockade of SCF activity by neutralizing antibody abrogated the efficacy of imatinib, imatinib is thought to inhibit growth of CD117<sup>+</sup> cells in an SCF-dependent manner. In fact, imatinib showed no effect on CD117<sup>-</sup> cells with low or faint SCF expression. This growth suppression by targeting CD117 was found to be mainly due to cell cycle arrest, not apoptosis induction in MFE280 cells (Supplementary Fig. 3A). However, since slight increase in apoptosis fraction was observed in Ishikawa cells by the treatment with imatinib (Supplementary Fig. 3B), imatinib may target both CD117-related and -unrelated pathways in a cell-type specific manner. Nevertheless, it is speculated that SCF plays a critical role as a mitogenic factor in the growth of CD117<sup>+</sup> cells, and the SCF/CD117 axis is a mechanical point on which imatinib exerts antitumor activities.

Considering the uses and potential effects of molecular target therapy, the percentage of CD117<sup>+</sup> cells in tumor burden should be concerning. Immunohistochemistry showed that the percentage of CD117<sup>+</sup> cells varied among patients, and that patients with



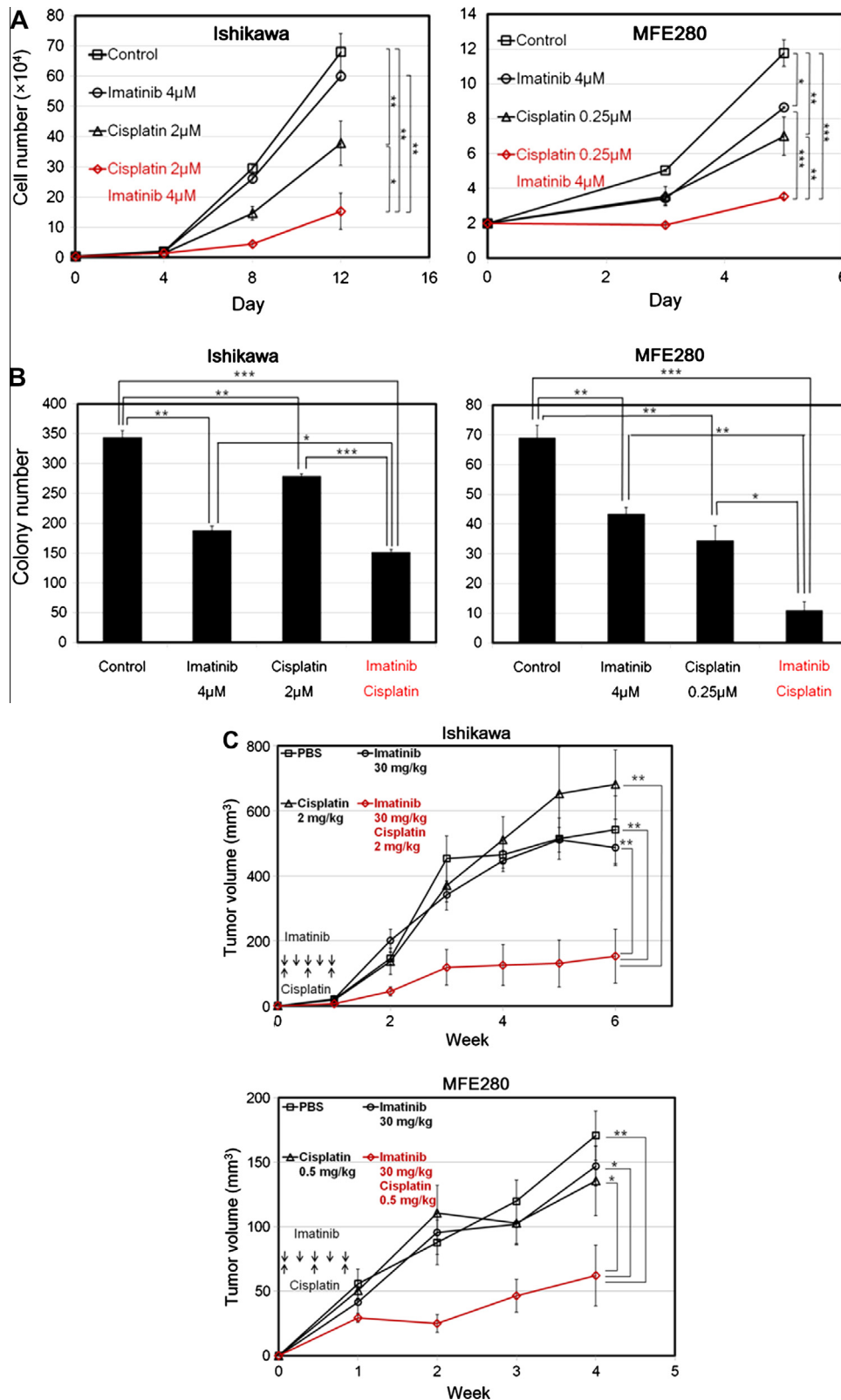
**Fig. 3.** Expression and role of SCF in endometrial cancer cells. (A) RT-PCR assay for CD117 and the CD117 ligand, stem cell factor (SCF) in FACS-sorted CD117<sup>+</sup> or CD117<sup>-</sup> Ishikawa cells. (B) Enzyme-linked immunosorbent assay (ELISA) measured the levels of SCF secretion in Ishikawa cells cultured in growth media in the absence or presence of anti-SCF antibody or control IgG. Data are the mean  $\pm$  S.D. in triplicate. (C) Clonogenic assay to examine the effects of anti-SCF antibody on colony-forming activity of Ishikawa cells. A total of  $2 \times 10^4$  Ishikawa cells were cultured in 6-cm dishes in the presence of anti-SCF antibody or control IgG for 6 days, and the colony number was counted. Data are the mean  $\pm$  S.D. in triplicate. \* $P < 0.01$ ; \*\* $P < 0.001$ .



**Fig. 4.** Effects of imatinib on endometrial cancer cells. (A) A total of  $2 \times 10^3$  FACS-sorted CD117<sup>+</sup> or CD117<sup>-</sup> cells were cultured in 96-well plates and treated with imatinib at indicated concentrations for 72 h, and the cell viability was examined by WST-1 assay. (B, C). A total of  $2 \times 10^3$  FACS-sorted CD117<sup>+</sup> (B) or CD117<sup>-</sup> (C) cells were cultured in 96-well plates and treated with imatinib at 8 μM in the presence of anti-SCF antibody or control IgG for 72 h, and the WST-1 assay was performed. Data are the mean  $\pm$  S.D. in triplicate. \* $P < 0.05$ ; \*\* $P < 0.01$ .

frequent CD117 expression were rare. Most of the cells that occupy tumors are therefore CD117<sup>-</sup> cells, which conventional chemotherapies should mainly target. Several clinical trials have been attempted with a single use of imatinib, both in recurrent gynecologic tumors and as a maintenance agent in patients following a complete clinical remission; however, all of them have demonstrated no or little efficacy [10,19]. These background results prompted us to attempt combination treatment with imatinib and cisplatin, based on the speculation that CD117-positive cancer stem-like cells are the source of more differentiated daughter cells that occupy the tumor, while CD117-negative cells fail to generate such cells. Targeting CD117<sup>+</sup> cells will therefore lead to depletion

of tumor-forming daughter cells while most of CD117<sup>-</sup> cells that initially occupy tumor are sensitive to conventional chemotherapy, thereby exhibiting enhanced growth inhibitory effect in combination. *In vitro* evaluation by assessing growth curve and colony formation revealed that imatinib at 4 μM or cisplatin at 2 μM had a modest effect in a single dose. However, combining them produced a significantly enhanced effect. It was of note that this combined effect was exaggerated *in vivo*. While a single dose of imatinib at 30 mg/kg or cisplatin at 2 mg/kg in mice showed minimal effects on tumor growth, combined administration achieved far more significant growth inhibition. Notably, growth inhibition appeared in a week after the final treatment with the agents, much earlier than



**Fig. 5.** Imatinib sensitizes endometrial cancer cells to cisplatin. (A) A total of  $3 \times 10^3$  Ishikawa or  $2 \times 10^4$  MFE280 cells were seeded in 24-well plates and treated with imatinib with or without cisplatin at the indicated concentrations for various days. The mean numbers of cells per well were counted and shown. Data are the mean  $\pm$  S.D. in triplicate. (B) A total of  $6 \times 10^4$  Ishikawa or  $5 \times 10^4$  MFE280 cells were seeded in 6-cm dishes and treated with imatinib and/or cisplatin at the indicated concentrations for 14 days. The colony numbers per well were counted and shown. Data are the mean  $\pm$  S.D. in triplicate. (C) Nude mice were inoculated subcutaneously into the right flanks with  $1 \times 10^7$  Ishikawa cells or  $1.5 \times 10^7$  MFE280 cells. After tumor cell inoculation, mice were randomly treated with PBS, cisplatin, imatinib, or the combination of cisplatin with imatinib. Each group consisted of 4 mice (for Ishikawa cells) or 6 mice (for MFE280 cells). Cisplatin (2 mg/kg for Ishikawa cells or 0.5 mg/kg for MFE280 cells), imatinib (30 mg/kg) and PBS were administered intraperitoneally on Days 1, 3, and 5, Days 1, 2, 3, 4, and 5 or Days 2 and 4, respectively. Tumor diameters were measured every week and are shown as mean  $\pm$  SE. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



expected. Six weeks of observation after starting the treatment revealed a prominent shrink in tumor volume (1/4–1/5) compared with controls or samples with single use of each agent. It is thus possible that the combination had a synergistic effect, rather than an additive effect. The fact that no significant effect on tumor growth was observed by a single use of imatinib alone might show a limitation of imatinib in targeting CD117<sup>+</sup> cells alone, leaving CD117<sup>−</sup> cells untargeted.

What are the mechanisms of the enhanced effect by using a combination *in vivo*? Cisplatin treatment is known to induce a variety of survival signalings, including hypoxia-inducible factor (HIF)-1 or Akt signalings [8,18,20]. HIF-1 induction is reported to induce SCF; SCF can up-regulate HIF-1 transcription, thereby forming a positive activation loop, which must promote angiogenesis *in vivo* [5,14,26]. Such a microenvironment will disturb the effect of cisplatin or induce resistance *in vivo*. The action of imatinib on the SCF/CD117 axis may target this positive loop, inhibiting tumor angiogenesis. In fact, the inhibitory effect of imatinib on tumor angiogenesis has been reported in other tumor types [14,23]. Additionally, the inhibitory effect of imatinib on CD117<sup>+</sup> cells is associated with the down-regulation of pro-survival signaling, such as Akt signalings [4,22], the representative signalings related to cisplatin resistance. These effects will contribute overall to enhancing the effects of cisplatin.

In summary, we identified CD117 as a marker of aggressive behavior of cells in endometrial cancer and as an independent prognostic factor for endometrial cancer. Imatinib inhibited the growth of CD117<sup>+</sup> cells in an SCF-dependent manner. Cisplatin treatment combined with imatinib conferred enhanced anti-tumor effects compared with a single use of each agent, probably inhibiting both CD117<sup>+</sup> and CD117<sup>−</sup> populations. Profound mechanistic analysis will be needed to uncover the molecular mechanisms of this enhanced anti-tumor effect for future clinical use. Furthermore, analysis of the combined effects of imatinib with other agents, such as taxanes, will provide greater opportunity for clinical application of imatinib in endometrial cancer.

## Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2013.11.020>.

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